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(74) Agents: BAUDE, Eric, J. et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, Eighth Floor, San Francisco, CA 94111-3834 (US).

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09/874,547 4 June 2001 (04.06.2001) US(71) Applicant (*for all designated States except US*): ZY-OMYX, INC. [US/US]; 26101 Research Road, Hayward, CA 94545 (US).

(72) Inventors; and

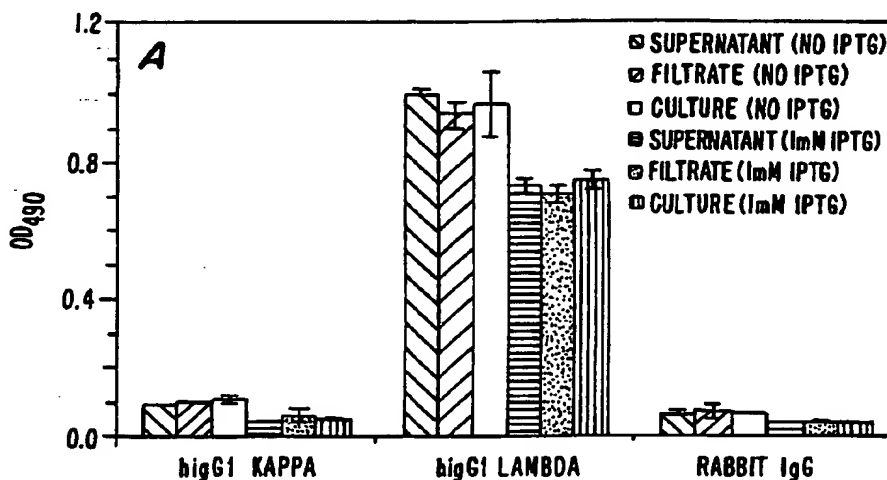
(75) Inventors/Applicants (*for US only*): NOCK, Steffen [DE/US]; 3269 Glenwood Avenue, Redwood City, CA 94062 (US). KASSNER, Paul, D. [US/US]; 247 W. 40th Avenue, San Mateo, CA 94403 (US).(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: SCREENING OF PHAGE DISPLAYED PEPTIDES WITHOUT CLEARING OF THE CELL CULTURE



(57) Abstract: This invention provides methods for screening populations of phage-displayed polypeptides that are particularly well-suited for high-throughput screening. The methods do not require the clearing of cells from a culture used to obtain the population of phage or other replicable genetic packages. Accordingly, the invention provides methods for forming complexes between a replicable genetic package displaying a polypeptide fusion and a target molecule in an uncleared cell culture containing replicable genetic package. Compositions made up of an uncleared cell culture containing replicable genetic packages displaying a polypeptide fusion and a target molecule are provided in the invention as well.



*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

<b>SCREENING OF PHAGE DISPLAYED PEPTIDES WITHOUT CLEARING OF THE CELL CULTURE</b>
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**CROSS-REFERENCES TO RELATED APPLICATIONS**

5                   This application claims the benefit of U.S. Provisional Patent application  
Serial No. 60/209503, filed on June 5, 2000, the teachings of which are herein  
incorporated by reference.

**STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER  
FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT**

10                   Not applicable.

**BACKGROUND OF THE INVENTION**

Phage display and related techniques have become powerful methods for  
15 the discovery of affinity binding reagents (Smith (1985) *Science* 228: 1315-1317). Linear  
and constrained peptides, antibody fragments (e.g., scFvs, Fvs and Fabs), as well as a  
number of alternative binding domains have all been displayed on phage particles, for  
example, via fusion to one of the phage coat proteins. Although several phage proteins  
(derived from gVIII, gVI, gVII and gIX) have all been used as fusion partners for display  
20 of recombinant proteins, gIII is the most widely used. Phagemids containing a phage  
origin of replication, an antibiotic resistance marker, and a gene encoding a binding  
domain/gIII fusion protein are readily constructed via conventional molecular biology  
techniques. Through large-scale ligation and transformation as well as recombination  
strategies, large libraries of  $10^8$  to  $10^{11}$  different recombinants are now being generated  
25 for use in affinity selection strategies (de Haard *et al.* (1999) *J. Biol. Chem.* 274: 18218-  
18230; Sblattero and Bradbury (2000) *Nat. Biotechnol.* 18: 75-80); Sheets *et al.* (1998)  
*Proc. Natl. Acad. Sci., U.S.A.* 95:6157-6162, published erratum appears in *Proc. Natl.*  
*Acad. Sci., U.S.A.* (1999) 96: 795).

Once a library of phage displaying potential binding agents is generated,  
30 individual phage with the capacity to bind to a chosen target must be isolated from an  
enormous excess of non-binding phage. To screen large numbers of phage to identify  
those that display polypeptides having a desired activity, it is desirable to develop high-  
throughput screening (HTS) methods. Preferably, such HTS methods would automate the

phage screening process so that large numbers of phage could be screened with little human intervention. Although HTS methods are available for many types of screening, previously known phage display protocols include steps that are not readily automatable. In particular, phage display protocols require, prior to screening, separation of the phage  
5 from the host cells in which the phage are amplified.

Traditionally, overnight cultures of bacteria producing phage are centrifuged or filtered to pellet bacteria and phage supernatants are used in the screening (See generally, Kay *et al.*, eds. (1996) *Phage display of peptides and proteins: a laboratory manual*. Academic Press Inc., San Diego CA). Alternatively, phage can be  
10 purified and concentrated from cleared supernatants by precipitation (e.g., with polyethylene glycol). However, these clearing methods are not readily performed by robotic systems (e.g., automated workstations). Therefore, time-consuming and expensive human intervention is required. These drawbacks are exacerbated as the numbers of samples are increased and during high-throughput screening. Therefore, a  
15 need exists for more fully automated methods for screening of phage display libraries. The present invention fulfills this and other needs.

#### SUMMARY OF THE INVENTION

The present invention provides methods for screening a population of replicable genetic packages (e.g., phage, eukaryotic viruses, and the like) to obtain  
20 particles that display on their surface a fusion protein that specifically binds to a target molecule. Unlike previous methods, which involve clearing a culture of cells prior to screening the methods of the present invention involve contacting a target molecule with an uncleared cell culture that contains a population of replicable genetic packages. Each replicable genetic package displays on its surface a fusion protein that has a surface-  
25 displayed replicable genetic package polypeptide and a potential binding polypeptide. The replicable genetic package that specifically bind to the target molecule form complexes containing replicable genetic packages and target molecules. In some cases, the potential binding polypeptide can be encoded by a member of a library of nucleic acid molecules. For example, the nucleic acid molecules can be cDNA molecules or  
30 recombinant products. In other cases, the potential binding polypeptide can be, for example, an antibody, or derivative of an antibody. For example, the potential binding polypeptide can be a scFv or a Fab.

The methods of the invention are useful for obtaining polypeptides that bind to essentially any molecule. For example, the target molecule can be a polypeptide,

an RNA, a DNA, a small organic molecule and a carbohydrate. The target molecules can be immobilized directly or indirectly to a solid support. Solid supports such as a bead, a chip, a microtiter plate, a eukaryotic cell, or a prokaryotic cell are present in some embodiments of the invention. The solid supports of the present invention can contain a variety of materials, such as Sepharose, polystyrene, glass, silicon oxide, etc.

In some embodiments, the methods also involve obtaining replicable genetic packages that specifically bind to the target molecule. For example, the replicable genetic packages that specifically bind to the target molecule can be separated from the bacterial cells after the binding of the phage to the target molecule. For example, the uncleared cell culture can be separated from a replicable genetic package-target complex(es) using aspiration. Once the replicable genetic packages are bound to the target molecule, some embodiments of the invention can further involve eluting the replicable genetic packages from the target molecule. Also, some embodiments involve identifying the replicable genetic packages that specifically bind to the target molecule with a detection reagent.

The present invention also provides compositions containing an uncleared cell culture, which contains: (a) a population of replicable genetic packages that display on their surfaces a fusion protein that includes a surface-displayed replicable genetic package polypeptide and a potential binding polypeptide; (b) a complex that is composed of one or members of the library of replicable genetic packages that specifically bind to the target molecule; and (c) cells in which the replicable genetic packages were amplified.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B show that the binding of HP6054-scFv and HP6054-Fab phage to human lambda light chain is not affected by the presence of bacteria in the sample. Antigens were immobilized on 96-well plates (Nunc, Denmark) at 10  $\mu$ g/ml. A phage ELISA was conducted using either an uncleared bacterial culture, or supernatants clarified by centrifugation or filtration. Each bar represents the mean  $\pm$  s.d. of duplicate samples. Figure 1A depicts representative results from the HP6054 scFv-phage, and Figure 1B is representative of HP6054 Fab-phage.

Figure 2 shows that the sensitivity of phage ELISA is not impaired by the presence of bacteria. Overnight cultures of HP6002 scFv-phage and HP6025 scFv-phage were mixed at various ratios and then supernatants or uncleared culture was tested in the

phage ELISA against hIgG2 and hIgG4 (each at 10 µg/ml). Each bar represents the mean  $\pm$  s.d. of duplicate samples.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

### 5 DEFINITIONS

“Replicable genetic packages” include virions of bacteriophage, filamentous phage, or any other eukaryotic virus, bacterial virus or phage. The term “phage” as used herein encompasses not only bacteriophage but also other types of replicable genetic packages, except where the term is used in a context that dictates a  
10 more specific meaning.

A molecule is “display(ed) on their surface” of a replicable genetic package if at least part of the molecule is accessible to the milieu surrounding a replicable genetic package.

The phrase “specifically (or selectively) binds to” in the context of a  
15 replicable genetic package refers to a binding reaction which is determinative of the presence of a replicable genetic package binding to a target molecule(s) in the presence of a population of other proteins, biologics, and replicable genetic packages. Thus, under designated binding conditions, a specifically binding replicable genetic package will bind to a particular molecule (e.g., target molecule) and under the same designated binding  
20 conditions, native replicable genetic packages do not bind to a particular molecule in a significant amount. Typically, a replicable genetic package “specifically” binds to a target molecule when the number of replicable genetic packages displaying a potential binding polypeptide that are bound to the target molecules is at least twice the background binding observed using a native replicable genetic package as a control.

25 An “uncleared cell culture” is an aqueous medium containing bacterial or eukaryotic cells. Typically, the “uncleared cell culture” is a growth of bacterial or eukaryotic cells infected with one or more replicable genetic package clones.

A “target molecule” is essentially any molecule that is being used as a probe to identify molecules that will bind to the target molecule. Examples of target  
30 molecules include, without limitation, amino acids, peptides, proteins, polypeptides, carbohydrates, small organic molecules, inorganic molecules, etc.

A “surface-displayed replicable genetic package polypeptide” is a polypeptide that is, at least in part, exposed to the milieu surrounding the virion.

Examples of "surface-displayed replicable genetic package polypeptides" include, without limitation, pIII and pVIII.

5 A "potential binding polypeptide" is a polypeptide that may possibly bind to the target molecule. A "potential binding polypeptide" can be screened for its ability to bind to a target molecule of choice.

A "replicable genetic package-target complex" is a complex in which a target molecule is bound to a replicable genetic package. The target molecule is bound to the replicable genetic package through the binding domain portion of a polypeptide displayed on the surface of a replicable genetic package.

10 An "antibody" can be derived from sequence of a mammal, non-mammal (e.g., birds, chickens, fish, etc.), or fully synthetic antibody sequences. A "mammal" is a member of the class Mammalia. Examples of mammals include, without limitation, humans, primates, chimpanzees, rodents, mice, rats, rabbits, sheep, and cows. The term "antibody" also refers to fragments and substitutes for antibodies such as F(ab')<sub>2</sub>, Fab',  
15 and Fab fragments. Additionally the "antibodies" can be single chain antibodies known as ScFv fragments, which are obtained by recombinantly fusing the variable regions of the light and heavy chains of the antigen binding fragment of interest.

## 20 I. INTRODUCTION

The present invention provides methods and compositions for screening replicable genetic package particles (e.g., phage, viruses, etc.) that display polypeptides for their ability to bind to a target molecule. Traditionally, such screening methods required clearing the host cells or bacteria from an uncleared cell culture and/or isolating a replicable genetic package stock before incubation with the target molecule. The  
25 methods of the present invention, however, do not require these procedures. Therefore, the invention provides significant advantages over previously available methods for screening phage and other particles, particularly when used in a high-throughput format.

Briefly, methods of the invention involve infecting bacteria or other suitable host cells with phage particles (or incubating cells that are transfected with a phagemid expression vector with helper phage) to generate an uncleared cell culture that  
30 contains a library of phage particles. This uncleared culture is then incubated with a target molecule. Phage particles that display a polypeptide that binds to the target molecule form a complex with the target molecule. After an incubation period, the bacterial or other cells used to amplify the phage can be separated from the phage

particles that bind to the target molecule. The phage particles that were able to bind to the target molecule can then be further purified, characterized, amplified, and/or detected, etc. These methods and compositions will be described in more detail below.

## 5 II. REPLICABLE GENETIC PACKAGE DISPLAY LIBRARIES

The methods of the invention are useful for screening a wide variety of phage display libraries. Phage display and related techniques provides a powerful method for selecting proteins of interest from large libraries (Bass *et al.* (1990) *Proteins: Struct. Funct. Genet.* 8: 309; Lowman and Wells (1991) *Methods: A Companion to Methods*  
 10 *Enz.* 3(3);205-216. Lowman and Wells (1993) *J. Mol. Biol.* 234;564-578). Each phage or other particle displays a unique variant protein on its surface and packages the gene encoding that particular variant. For example, the libraries can be composed of homogenous or heterogenous populations of phage particles. That is, each phage in the library can display the same potential binding polypeptide, or each phage can display a  
 15 different potential binding polypeptide. Potential binding polypeptides can serve as epitopes, ligands, agonists, antagonists, enzymes, etc. For example, the potential binding polypeptides can encode scFvs and Fabs.

Some recent reviews on the phage display technique include, for example, McGregor (1996) *Mol Biotechnol.* 6(2):155-62; Dunn (1996) *Curr. Opin. Biotechnol.*  
 20 7(5):547-53; Hill *et al.* (1996) *Mol Microbiol* 20(4):685-92; *Phage Display of Peptides and Proteins: A Laboratory Manual*. BK. Kay, J. Winter, J, McCafferty eds., Academic Press 1996; O'Neil *et al.* (1995) *Curr. Opin. Struct. Biol.* 5(4):443-9; Phizicky *et al.* (1995) *Microbiol. Rev.* 59(1):94-123; Clackson *et al.* (1994) *Trends Biotechnol.* 12(5):173-84; Felici *et al.* (1995) *Biotechnol. Annu. Rev.* 1:149-83; Burton (1995)  
 25 *Immunotechnology* 1(2):87-94.) See, also, Cwirla *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 6378-6382 (1990); Devlin *et al.*, *Science* 249: 404-406 (1990), Scott & Smith, *Science* 249: 386-388 (1990); Ladner *et al.*, US 5,571,698.

The methods of the invention are applicable to any of the genetic packages most frequently used for phage display libraries. These include, for example,  
 30 bacteriophage, particularly filamentous phage, and especially phage M13, Fd and F1. (Webster (1996) Chapter 1, *Biology of the Filamentous Bacteriophage*, in Kay *et al.*, eds. (1996) *Phage Display of Peptides and Proteins*). Microbiological methods for growing, titrating, and preparing filamentous phage particles, and phage DNA are known in the art (Rider *et al.* (1996) Chapter 4, *Microbiological Methods*, in Kay *et al.*, eds. (1996)) and



their genomes are very well characterized. These filamentous phage have genes which encode the various capsid proteins and are known as genes III, VI, VII, VIII, and IX (Webster *et al.*, (1996), *supra*). The proteins the genes encode are known as pIII, pVI, pVII, pVIII, and pIX, respectively. The most abundant capsid protein is pVIII, which has 2700 copies on the surface of the phage. Approximately 5 copies of pIII are displayed on the phage particle.

Typically, libraries of nucleic acid molecules are ligated into a phage-display vector and introduced into bacteria to create a library of particles displaying fusion proteins that consist of a surface-displayed phage polypeptide and a potential binding polypeptide. Most work has involved inserting nucleic acid libraries encoding polypeptides to be displayed into either a gIII or gVIII expression vector in order to produce phage-displayed fusion protein(s) (See, e.g., Dower, WO 91/19818; Devlin, WO 91/18989; MacCafferty, WO 92/01047 (gene III); Huse, WO 92/06204; Kang, WO 92/18619 (gene VIII)). These fusion proteins generally included a signal sequence, usually but not necessarily, from the phage coat protein, a polypeptide to be displayed and either the gene III or gene VIII protein or a fragment thereof. Exogenous coding sequences are often inserted at or near the N-terminus of gene III or gene VIII, although other insertion sites are possible. pVIII, however, can only tolerate short inserts – about 5 to 6 amino acid residues. (Armstrong *et al.*, (1996), *supra*). Larger peptides can be displayed as pVIII fusions if pVIII wild-type coat proteins are interspersed with the recombinant pVIII (Malik *et al.* (1996) Chapter 8, *Multiple Display of Foreign Peptide Epitopes on Filamentous Bacteriophage Virions*, in Kay *et al.*, eds. (1996)).

A variety of vectors for displaying pIII and pVIII fusion proteins in a phage display library have been described (Armstrong *et al.* (1996) Chapter 3, *Vectors for Phage Display*, in Kay *et al.*, eds. (1996); Dottavio (1996) Chapter 7, *Phagemid-Displayed Peptide Libraries*, in Kay *et al.*, eds. (1996); (Malik *et al.* (1996) Chapter 8, *Multiple Display of Foreign Peptide Epitopes on Filamentous Bacteriophage Virions*, in Kay *et al.*, eds. (1996)) and are commercially available (e.g., pCANTAB5E, Pharmacia; λSurfZap, Stratagene).

Eukaryotic replicable genetic packages such as eukaryotic viruses can also be used to display polypeptides in an analogous manner. For example, display of human heregulin fused to gp70 of Moloney murine leukemia virus has been reported by Han *et al.*, (1995) *Proc. Nat'l. Acad. Sci. USA* 92: 9747-9751.

Alternatively, prokaryotic spores can be used as replicable genetic packages. In this case, polypeptides are displayed from the outer surface of the spore. For example, spores from *B. subtilis* have been reported to be suitable. Sequences of coat proteins of these spores are described in Donovan *et al.*, *J. Mol. Biol.* 196: 1-10 (1987).

5 Thus, spores can be used to display the potential binding polypeptides.

The nucleic acid libraries encoding the potential binding polypeptides can be constructed from nucleic acids from a variety of sources, including cDNA, genomic DNA, synthetic nucleotides and/or from oligomers encoding randomized peptides (see, e.g., Adey *et al.* (1996) Chapter 5, *Construction of Random Peptide Libraries in Bacteriophage M13*, in Kay *et al.*, eds. (1996) for descriptions of randomized peptide libraries). Random peptide libraries have been constructed using synthetic degenerate oligonucleotides and expressed as fusions with pIII (Adey *et al.*, (1996), *supra*). Also, libraries of antibody and antibody fragments (Fv, scFv and Fab) can be expressed in phage display systems with pIII (McCafferty and Johnson (1996) Chapter 6, 10 *Construction and Screening of Antibody Display Libraries*, in Kay *et al.*, eds. (1996)). One method of constructing an antibody phage display library involves generating nucleic acids encoding antibody fragments from the amplification of variable domain gene sequences (McCafferty and Johnson (1996), *supra*). The fragments can be amplified from nucleic acids isolated from antigen immunized or non-immunized sources. The 15 nucleic acids encoding variable heavy and light chain domains are then spliced together using overlap PCR and ligated into a phage-display vector to subsequently generate the antibody phage display library (McCafferty and Johnson (1996), *supra*). 20

Molecular biological methods that can be used to isolate, manipulate, and generate the nucleic acid libraries of the present invention are well known in the art and are detailed in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2<sup>nd</sup> ed. 1989); 25 Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., (1994)).

Numerous other methods for constructing phage display libraries are known in the art. For example, libraries expressing fragments of a protein can be used to 30 map the epitopes of an antibody, which can serve as the target molecule (Plessis and Jordaan (1996) Chapter 9, *Phage Libraries Displaying Random Peptides Derived from a Target Sequence*, in Kay *et al.*, eds. (1996)). Also, once a recombinant phage has been isolated or constructed, it can be used to construct a second-generation phage-display

library through DNA shuffling (Adey *et al.* (1996) Chapter 16, *Preparation of Second-Generation Phage Libraries*, in Kay *et al.*, eds. (1996)).

Once the nucleic acids have been introduced into an appropriate expression vector, phage particles are obtained. The vectors are introduced into appropriate host cells and amplified. Uncleared cell cultures containing libraries of phage particles can be generated using methods well known in the art (see, e.g., Sparks *et al.* (1996) Chapter 13, *Screening Phage-Displayed Random Peptide Libraries*, in Kay *et al.*, eds. (1996)). For example, libraries of phage particles displaying the potential binding polypeptides can be used to infect bacteria (e.g., *E. coli*) in order to generate an uncultured cell culture. Alternatively, a library of nucleic acid molecules encoding the potential phage binding polypeptides (e.g., phagemid vectors) can be introduced into bacteria, which are subsequently infected with a helper phage (see, e.g., Sparks *et al.*, (1996) Ch. 13, *supra*). These procedures can generate a library of phage particles in an uncultured cell culture.

### III. SCREENING REPLICABLE GENETIC PACKAGE-DISPLAY LIBRARIES

Phage display libraries are screened to obtain phage that display on their surfaces a polypeptide that has a desired activity (e.g., the ability to bind to a target molecule). Methods for screening phage-displayed libraries are known in the art (Sparks *et al.* (1996) Chapter 13, *supra*); McCafferty and Johnson (1996) Chapter 6, *supra*; McCafferty (1996) Chapter 15, *Phage Display: Factors Affecting Panning Efficiency*, in Kay *et al.*, eds. (1996)). To date, however, these methods involve either clearing a cell culture (e.g., by centrifugation, filtration) or isolating the entire phage library in the culture (e.g., by precipitation, centrifugation, etc.) for subsequent screening. This represents an extra step that necessitates the expenditure of extra time and effort to transfer the container or plate containing the uncultured cell culture to another format suitable for centrifugation, filtration, etc. For example, centrifugation of uncultured cell cultures that have been transferred to or grown in a microtiter plate requires transferring the plate to a centrifuge. This requires an operator to move the plate from the bench to the centrifuge, wait for the centrifugation to take place, and then remove the cleared culture from the plate to continue with the screening. These time consuming and unnecessary steps for clarifying a bacterial culture in the screening of a phage display library can be eliminated using the methods of the present invention.

Screening involves selecting phage that display on their surface a polypeptide that has a desired biological activity. Often screening entails identifying phage whose potential binding polypeptides can bind to a target molecule. In general, enough clones or pfu should be screened to ensure an adequate representation of displayed peptides is being screened. Preferably about  $10^5$ - $10^6$  pfu would be screened, more preferably at least about  $10^9$  pfus would be screened, still more preferably at least about  $10^{11}$ - $10^{12}$  pfu, would be screened. Often more than one round of screening will be necessary to identify or sufficiently enrich the phage particles of interest.

Suitable target molecules include a wide variety of molecules and include a molecule for which a practitioner desires to identify or isolate a polypeptide that will bind to the target molecule. For example, the target molecule can be an antigen where a library of phage displaying antibodies (e.g., scFv or Fab) are being screened to identify antibody sequences that bind to that particular antigen. Thus, a variety of targets (e.g., peptides, proteins, carbohydrates, nucleic acids, peptide nucleic acids, RNA, DNA, small organic molecules (i.e., carbon containing molecules of 100 kDa or less, more preferably 50 kDa or less, still more preferably 10 kDa or less), inorganic molecules, etc.) can be used to probe a phage-display library. Essentially, the target can be any substance that can serve as a ligand for the potential binding polypeptide of the phage-displayed polypeptide. If possible, a positive control for the retention of binding activity of the target for a potential binding polypeptide of interest should be included in the screening process to ensure proper conditions for identifying the phage are maintained.

The immobilization of a target or target-binding molecule to a solid support can facilitate separation of replicable genetic packages that can bind to the target molecule from the cells and unbound replicable genetic packages that are present in the uncleared cell culture. One or more species of target molecules can be immobilized directly or indirectly as an array (i.e., a two or three-dimensional arrangement of molecules) on a solid support.

Those of skill in the art will recognize a variety of methods to immobilize a target molecule to a solid support. For example, the target molecule(s) can be directly or indirectly immobilized on a solid support (see below). The target molecule can be immobilized directly to the solid support through covalent and non-covalent bonds.

Alternatively, the target molecule can be indirectly bound to the solid support by coating the solid support with a substance or molecule that can bind to the target molecule. For example, the solid support can be coated with streptavidin and the

target molecule can be biotinylated (Sparks *et al.* (1996), *supra*). Thus, the biotinylated target molecules can be immobilized to the strepavidin coated solid support through the biotin-strepavidin interaction. Those of skill in the art will also recognize that immobilized metal affinity substrates can be used in the present invention to indirectly  
5 bind the target molecule to a solid support (see Ausubel *et al.*, eds., (1994) for review of immobilized metal affinity technology). For example, solid supports containing Ni-NTA (nickel-nitrilotriacetic acid) such as Ni-NTA Agarose (Qiagen) or Ni-NTA Magnetic Agarose Beads (Qiagen) can be used to bind target molecules having an N-terminal or C-terminal stretch of poly-histidine (e.g., 6 or more histidines). Ni-NTA Magnetic Agarose  
10 Beads are beads of agarose, containing magnetic particles and nitrilotriacetic acid (NTA) groups on their surfaces. The replicable genetic package-target molecule complexes can be released from a Ni-NTA substrate by an increase in the concentration of an imidazole in the solution sufficient to disrupt the poly-histidine-Ni-NTA interaction. Other suitable methods of indirectly immobilizing target molecules include the binding of a target  
15 having a ligand binding protein moiety to a support that contains a ligand for the binding protein, e.g., maltose binding protein and amylose (New England Biolabs); an antibody with an Fc domain and protein A (Sparks *et al.* (1996), *supra*); and glutathione-S-transferase and glutathione agarose (see e.g., Ausubel *et al.*, eds., (1994), *supra*).

Alternatively the target can be soluble, i.e., not immobilized on a solid  
20 support. The uncleared cell culture is then incubated with the soluble target. Any resulting replicable genetic package-target complexes can subsequently be captured on a solid support by a target-binding molecule (see, e.g., Sparks *et al.* (1996) Ch. 13, *supra*; see also, methods for indirectly binding a target molecule above).

After immobilizing the target on the solid support, non-specific binding of  
25 phage to the solid support can be decreased with agents such as non-fat dry milk or BSA (bovine serum albumin). Those of skill in the art will recognize other agents that can be used alone or in combination to decrease non-specific binding such as a non-ionic detergent (e.g., Tween-20 or Triton-X-100).

A variety of solid supports can be used in the present invention. Examples  
30 of solid supports include, without limitation, bead, microtiter plates, chips, prokaryotic and eukaryotic cells. Beads can be composed of materials such as Sepharose, agarose, polystyrene, etc. and can be paramagnetic. Microtiter plates are commercially available in a variety of formats (e.g., 96, 384 and 1536 well plates) and materials (e.g., polystyrene). Chips can be comprised of a variety of materials, layers and substrates (see,

e.g., WO 00/04389). For example, substances for use solid supports can be selected from a group consisting of silicon, silica, quartz, glass, controlled pore glass, carbon, alumina, titania, tantalum oxide, germanium, silicon nitride, zeolites, and gallium arsenide. Many metals such as gold, platinum, aluminum, copper, titanium, and their alloys are also options for solid supports of the present invention. In addition, many ceramics and polymers may also be used as solid supports. Polymers which may be used as solid supports include, but are not limited to, the following: polystyrene; poly(tetra)fluoroethylene (PTFE); polyvinylidenedifluoride; polycarbonate; polymethylmethacrylate; polyvinylethylene; polyethyleneimine; poly(etherether)ketone; polyoxymethylene (POM); polyvinylphenol; polylactides; polymethacrylimide (PMI); polyatkenesulfone (PAS); polypropylene; polyethylene; polyhydroxyethylmethacrylate (HEMA); polydimethylsiloxane; polyacrylamide; polyimide; and block-copolymers. The solid support on which the target resides may also be a combination of any of the aforementioned solid support materials. The solid support can also be comprised of a eukaryotic or prokaryotic cell.

#### IV. SEPARATING THE REPLICABLE GENETIC PACKAGE-TARGET COMPLEX(ES) FROM THE UNCLEARED CELL CULTURE

If a population of phage or other particles include members that can specifically bind to a target molecule, those particles will bind to, and form a complex with, the target molecule. It is often desirable to remove the unbound components of the uncleared cell culture from complex (for example, removing the phage particles that do not specifically bind to the target molecule, the cells, and other components of the uncleared cell culture can be removed). In cases where the target is immobilized, directly or indirectly, on a solid support, the uncleared culture can be separated from the replicable genetic package-target complex using variety of separation methods known in the art. There are many separation methods known in the art (e.g., filtering, sedimenting, centrifuging, decanting, precipitation, etc.) that can be used or adapted for use in the present invention. For example, the where the target is immobilized on a microtiter plate, the uncleared cell culture can be aspirated from the well, leaving behind those replicable genetic packages that are bound to the immobilized target. Alternatively, the target can be immobilized on a bead and the uncleared cell culture can be passed through a filter with a pore size smaller than the bead, but larger than a bacterial cell (or a eukaryotic cell when using eukaryotic host cells). Another separation method is the immobilization of a

target on a paramagnetic bead, and the decantation of the uncleared cell culture leaving the replicable genetic package-target molecule complex behind bound to the paramagnetic bead held in place with a magnetic field.

If a target is used that is free in solution, any resulting replicable genetic package-target molecule complex(es) can be subsequently separated from the uncleared cell culture. For example, the replicable genetic package-target complex can be incubated in the presence of a third molecule, a target complex-binding molecule, that is immobilized on a solid support and does not disrupt the replicable genetic package-target molecule complex(es). The target complex-binding molecule can bind to either the soluble target molecule or to the replicable genetic package. This permits the replicable genetic package-target complex to bind to the target-binding molecule, thereby indirectly immobilizing the replicable genetic package-target complex. The uncleared cell culture can then be separated from the replicable genetic packages that bind specifically to the target molecule using the separation methods described above for the first category of replicable genetic package-target complexes.

In preferred embodiments, at least 70% of the cells are separated from the replicable genetic packages that are specifically bound to the target molecule, more preferably, at least 80% of the cells are separated from the replicable genetic packages that are specifically bound to the target molecule, still more preferably, at least 90% of the cells are separated from the replicable genetic packages that are specifically bound to the target molecule, yet still more preferably, substantially all of the bacterial cells are separated from the replicable genetic packages that are specifically bound to the target molecule.

It is sometimes desirable to wash the replicable genetic package-target complex. The wash can remove undesirable components of the cell cultures from the specifically bound replicable genetic packages. The wash can remove cells, non-specifically bound replicable genetic packages, etc. Often, a wash buffer is used. The wash buffer can contain a detergent, or other agents, and compositions that are compatible with replicable genetic package-target binding to increase the stringency of the screening process. For example, a wash buffer that can be used in the present invention is a solution of Tris buffered saline with 0.05% Tween-20, pH 7.4 (TBST).

For some applications, it is desirable to elute the replicable genetic packages that specifically bind to the target molecule. The replicable genetic packages can then be used for, for example, further rounds of screening, amplification, detection, or

characterization (e.g., nucleic acid sequencing). Elution can be accomplished using a variety of methods known in the art. The replicable genetic packages can be eluted using pH changes, protein denaturants, or EGTA/EDTA if a metal ion is necessary for replicable genetic package-target interaction (See e.g., Sparks *et al.* (1996) Ch. 13, *supra* for elution techniques using phage-display). For example, the replicable genetic packages can be eluted using an acidic buffer (e.g., glycine-HCl, pH 2) (see, e.g., Sparks *et al.* (1996) Ch. 13, *supra*). The eluate can then be removed and neutralized with the addition of a second buffer (e.g., NaPO<sub>4</sub> buffer pH 7.5) (see, e.g., Sparks *et al.* (1996) Ch. 13, *supra*). Alternatively, natural or synthetic ligands that interrupt the replicable genetic package-target complex can be used to elute the replicable genetic package from the target (Sparks *et al.* (1996) Ch. 13, *supra*).

If desired, the replicable genetic package(s) can be amplified in order to increase the number of replicable genetic package, thus potentially increasing the chance that enough of the replicable genetic package(s) will be present in the next round for isolation, identification, or detection, etc. Methods for amplifying replicable genetic packages in solid and liquid culture are known in the art (see, e.g., Sparks (1996), Ch. 13, *supra*, and Rider *et al.* (1996) Ch. 4, *supra*, for methods of amplifying filamentous bacteriophage).

Those of skill in the art will recognize that screening methods of the present invention can be optimized. Furthermore, skilled artisans will recognize methods of optimizing to determine the effectiveness of steps and to increase the chances of identifying the replicable genetic package of interest. For example, the inclusion of positive and negative controls in the screening process can facilitate the trouble-shooting and/or optimization of a screening process.

25

#### V. DETECTION AND CHARACTERIZATION OF REPLICABLE GENETIC PACKAGES

Another aspect of the present invention is that the presence of cells and other components of an uncleared culture do not interfere with the detection of particles that specifically bind to a target molecule. Accordingly, some methods of the present invention involve contacting the particle-target molecule complex with a detection reagent prior to removing the cells and/or other components of the uncleared cell culture.

30

The presence of replicable genetic packages that bind to a target molecule can be detected using a variety of materials and methods known to those of skill in the art.



For example, the replicable genetic package-target complexes can be incubated with a detection reagent. Typically, a detection reagent is labeled with a substance that permits the qualitative or quantitative determination of the presence or absence of the replicable genetic package-target complex. The term "labeled" refers to a composition is that is  
5 detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radiolabels (e.g.,  $H^3$ ,  $C^{13}$ ,  $C^{14}$ ,  $P^{32}$ ,  $S^{35}$ ,  $I^{125}$ ), fluorescent dyes, fluorophores, electron-dense reagents, enzymes and their substrates (e.g., as commonly used in enzyme-linked immunoassays, e.g., alkaline phosphatase and horse radish peroxidase), biotin-streptavidin, digoxigenin,  
10 or haptens and proteins for which antisera or monoclonal antibodies are available. The label or detectable moiety is typically bound, either covalently, through a linker or chemical bound, or through ionic, van der Waals or hydrogen bonds to the molecule to be detected.

Detection reagents can include antibodies – such as antibodies that react  
15 with the native form of the phage being used, e.g., anti-M13KO7 antibody. The antibody itself can be labeled. For example, horseradish peroxidase (HRP) can be conjugated to an anti-M13 antibody (Amersham-Pharmacia Biotech, Piscataway, NJ). The absorbance of the reaction produce of HRP and o-Phenylenediamine Dihydrochloride (OPD; Sigma, St. Louis MO) can be monitored with a 490 nm filter (Biorad, Hercules CA) after stopping  
20 the reaction with acid.

The detection of the replicable genetic package-target complex on the chip could be analyzed using a physical spectroscopy method, such as mass spectroscopy or surface plasmon resonance (U.S. Patent No. 5,641,640). Surface plasmon resonance has been used to detect phage-displayed antibody-target interactions (de Haard *et al.*, (1999)).  
25 Chips and surface plasmon resonance instruments are commercially available (e.g., BIACORE, Uppsala, Sweden) for the detection of analytes.

Fluorescence polarization could also be employed by modifying the target molecule with an appropriate fluorescence label or fluorophore (Burke *et al.* (1996) Chapter 18, *Measurement of Peptide Binding Affinities Using Fluorescence Polarization*,  
30 in Kay *et al.*, eds. (1996)).

The replicable genetic package that are bound to a target can be further characterized as to the genetic or protein makeup of their potential binding polypeptide(s). In some embodiments, the nucleic acid sequence of the potential binding polypeptide can be determined by sequencing the phagemid vector contained in a

particular phage (see e.g., Masecar *et al.* (1996) Chapter 17, *Nonradioactive Sequencing of Random Peptide Recombinant Phage*, in Kay *et al.*, eds. (1996)). The protein makeup of a phage could be determined using methods known in the art, such as immunological assays (e.g., Western blots), two-dimensional gels, mass spectrometry, etc.

5

#### VI. HIGH-THROUGHPUT SCREENING ON AN AUTOMATED WORKSTATION.

In the present invention, high-throughput analysis and screening of replicable genetic package-display libraries can be performed on a automated workstation (see e.g., U.S. Patent No. 5,139,744, "Automated laboratory workstation having module identification means."). An "automated workstation" is typically a computer-controlled apparatus which can, through robotic functions, transfer, mix, and remove liquids from microtiter plates. An automated workstation can also contain a built-in plate reader, which can read the absorbance of a liquid in a microtiter well. The automated workstation can be programmed to carry out a series of mixing, transfer, and/or removal steps. The automated workstation will typically have a multi-channel pipettor which can pipette small amounts of liquid (e.g., microliter amounts) from a vessel to the well.

For example, in some embodiments of the present invention, the automated workstation can transfer uncleared cell culture(s) into a micro-titer plate. The microtiter plate can have pre-immobilized target molecule(s) already in the wells. The automated workstation can subsequently be used to remove uncleared cell cultures from the wells, wash the wells, or elute the replicable genetic packages from the immobilized target. Detection of a replicable genetic package bound to an immobilized target molecule can also be carried out using an automated workstation. The automated workstation can be used to add a detection reagent to the wells. The automated workstation, when equipped with a plate reader, can monitor the absorbance of the reaction of the detection reagent in the wells.

#### EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

**Example 1 – Comparison of detection of phage from uncleared bacterial cultures and cleared bacterial cultures.**

This Example describes experiments in which alternatives to centrifugation or filtration prior to the screening of a phage-display library were explored.

- 5 Phage displaying scFvs or Fabs were generated by PCR amplification of cDNA corresponding to the heavy and light chain variable regions from the HP6002, HP6025, and HP6054 hybridomas (Reimer *et al.* (1984) *Hybridoma* 3: 263-275) (cells obtained from ATCC Manassas, VA; CRL-1788, CRL-1775 and CRL-1763 respectively). The regions were amplified using the primers (SEQ ID NOS: 1-84) set out in Table 1:

Table 1

## Primer sequences for ScFv and Fab library generation

Name	Mer	Sequence
MCH1-G1R	48	ATTGGCGCGCCTTATTAACAATCCCTGGGCACAATTTTCTTGTCACC
MCH1-G2A	44	ATTGGCGCGCCTTATTAACAGGGCTTGATTGTGGGCCCTCTGGG
MCH1-G2B	45	ATTGGCGCGCCTTATTAACAGGGGTTGATTGTTGAAATGGGCCCCG
MHV-Back1	50	TTATTACTCGCGGCCAGCCGGCCATGGCCGATGTGAAGCTTCAGGAGTC
MHV-Back2	50	TTATTACTCGCGGCCAGCCGGCCATGGCCAGGTGCAGCTGAAGGAGTC
MHV-Back3	50	TTATTACTCGCGGCCAGCCGGCCATGGCCAGGTGCAGCTGAAGCAGTC
MHV-Back4	50	TTATTACTCGCGGCCAGCCGGCCATGGCCAGGTACTCTGAAAGAGTC
MHV-Back5	51	TTATTACTCGCGGCCAGCCGGCCATGGCCGAGGTCCAGCTGCAACAATCT
MHV-Back6	50	TTATTACTCGCGGCCAGCCGGCCATGGCCGAGGTCCAGCTGCAGCAGTC
MHV-Back7	51	TTATTACTCGCGGCCAGCCGGCCATGGCCAGGTCCAACTGCAGCAGCCT
MHV-Back8	50	TTATTACTCGCGGCCAGCCGGCCATGGCCGAGGTGAAGCTGGTGGAGTC
MHV-Back9	50	TTATTACTCGCGGCCAGCCGGCCATGGCCGAGGTGAAGCTGGTGGAAATC
MHV-Back10	50	TTATTACTCGCGGCCAGCCGGCCATGGCCGATGTGAACTTGAAGTGTC
MHV-For1	33	ACCTGGCGCGCCTGCAGAGACAGTGACCAGAGT
MHV-for1b	42	ACCGCCTCCACCTGGCGCGCCTGCAGAGACAGTGACCAGAGT
MHV-For2	33	ACCTGGCGCGCCTGAGGAGACTGTGAGAGTGGT
MHV-for2b	42	ACCGCCTCCACCTGGCGCGCCTGAGGAGACTGTGAGAGTGGT
MHV-For3	33	ACCTGGCGCGCCTGAGGAGACGGTGACTGAGGT
MHV-for3b	42	ACCGCCTCCACCTGGCGCGCCTGAGGAGACGGTGACTGAGGT
MHV-For4	33	ACCTGGCGCGCCTGAGGAGACGGTGACCGTGGT
MHV-for4b	42	ACCGCCTCCACCTGGCGCGCCTGAGGAGACGGTGACCGTGGT
MKV-back1	39	TCTGGCGGTGGCGGATCGGATGTTTTGATGACCCAACT
MKV-Back2	39	TCTGGCGGTGGCGGATCGGATATTGTGATGACGCAGGCT
MKV-Back3	36	TCTGGCGGTGGCGGATCGGATATTGTGATAACCCAG
MKV-Back4	39	TCTGGCGGTGGCGGATCGGACATTGTGCTGACCCAATCT
MKV-Back5	39	TCTGGCGGTGGCGGATCGGACATTGTGATGACCCAGTCT
MKV-Back6	39	TCTGGCGGTGGCGGATCGGATATTGTGCTAACTCAGTCT
MKV-Back7	39	TCTGGCGGTGGCGGATCGGATATCCAGATGACACAGACT
MKV-Back8	39	TCTGGCGGTGGCGGATCGGACATCCAGCTGACTCAGTCT
MKV-Back9	39	TCTGGCGGTGGCGGATCGCAAATTGTCTCACCAGTCT
MKV-For1	38	GATGGTGATGTGCGGCCGCGCGTTTCAGCTCCAGCTTG

MKV-For2	40	GATGGTGATGTGCGGCCGCCCCGTTTTATTTCCAGCTTGGT
MKV-For3	39	GATGGTGATGTGCGGCCGCCCCGTTTTATTTCCAACTTTG
MKV-For4	40	GATGGTGATGTGCGGCCGCGGATACAGTTGGTGAGCATC
MVH1	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTRMAGCTTCAGGAGTCAGGA C
MVH2	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTSCAGCTKCAGCAGTCAGGA C
MVH3	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGCAGCTGAAGSASTCAGG
MVH4	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGCAGCTTCAGGAGTCSGGA C
MVH5	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGARGTCCAGCTGCAACAGTCYGGA C
MVH6	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTCCAGCTKCAGCAATCTGG
MVH7	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGSTBCAGCTGCAGCAGTCTGG
MVH8	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTTCAGCTGCAGCAGTCTGGR C
MVH9	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTTCAGCTGCAGCAGTCTGG
MVH10	56	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTCCARCTGCAACAATCTGGA CC
MVH11	54	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTCCACGTGAAGCAGTCTGGG
MVH12	52	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGAASSTGGTGAATCTG
MVH13	52	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAVGTGAAGYTGGTGGAGTCTG
MVH14	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGCAGSKGGTGGAGTCTGGG G
MVH15	54	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAKGTGCAMCTGGTGCAGTCTGGG
MVH16	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGAAGCTGATGGARTCTGG
MVH17	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGCARCTTGTGAGTCTGGT G
MVH18	54	CCTTTCTATGCGGCCAGCCGGCCATGGCCGARGTRAAGCTTCTCGAGTCTGGA
MVH19	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAAGTGAARSTTGAGGAGTCTGG
MVH20	54	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAAGTGATGCTGGTGGAGTCTGGG
MVH21	55	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGTTACTCTRAAGWGTSTGGC C
MVH22	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTCCAAC TVCAGCARCCTGG
MVH23	52	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTTCARCTGCAGCAGTCTG
MVH24	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGATGTGAAC TTGGAAGTGTCTGG
MVH25	53	CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGAAGGTCATCGAGTCTGG

MVK1	38	TTACTCCGGTCCGCGGACATTGTTCTCACCCAGTCTCC
MVK2	38	TTACTCCGGTCCGCGGACATTGTGCTSACCCAGTCTCC
MVK3	38	TTACTCCGGTCCGCGGACATTGTGATGACTCAGTCTCC
MVK4	38	TTACTCCGGTCCGCGGACATTGTGCTMACTCAGTCTCC
MVK5	38	TTACTCCGGTCCGCGGACATTGTGYTRACACAGTCTCC
MVK6	38	TTACTCCGGTCCGCGGACATTGTRATGACACAGTCTCC
MVK7	38	TTACTCCGGTCCGCGGACATTMAGATRACCCAGTCTCC
MVK8	38	TTACTCCGGTCCGCGGACATTCAGATGAMCCAGTCTCC
MVK9	38	TTACTCCGGTCCGCGGACATTCAGATGACDCAGTCTCC
MVK10	38	TTACTCCGGTCCGCGGACATTCAGATGACACAGACTAC
MVK11	38	TTACTCCGGTCCGCGGACATTCAGATCATTAGTCTCC
MVK12	38	TTACTCCGGTCCGCGGACATTGTTCTCAWCCAGTCTCC
MVK13	38	TTACTCCGGTCCGCGGACATTGTTCTCTCCCAGTCTCC
MVK14	38	TTACTCCGGTCCGCGGACATTGWGCTSACCCAATCTCC
MVK15	37	TTACTCCGGTCCGCGGACATTSTGATGACCCARTCTC
MVK16	38	TTACTCCGGTCCGCGGACATTKTGATGACCCARACTCC
MVK17	38	TTACTCCGGTCCGCGGACATTGTGATGACTCAGGCTAC
MVK18	38	TTACTCCGGTCCGCGGACATTGTGATGACBCAGGCTGC
MVK19	37	TTACTCCGGTCCGCGGACATTGTGATAACYCAGGATG
MVK20	38	TTACTCCGGTCCGCGGACATTGTGATGACCCAGTTTCG
MVK21	38	TTACTCCGGTCCGCGGACATTGTGATGACACAACCTGC
MVK22	38	TTACTCCGGTCCGCGGACATTTTGCTGACTCAGTCTCC
MVK23	38	TTACTCCGGTCCGCGGACATTTTGCTGACTCAGTCTCC
MVK24	38	TTACTCCGGTCCGCGGACATTGTAATGACCCAATCTCC
MVK25	38	TTACTCCGGTCCGCGGACATTGTGATGACCCACACTCC

Assembled scFv or Fab DNA sequences were digested with SfiI and NotI, subcloned into the pCANTAB5E vector (Amersham-Pharmacia Biotech, Piscataway, NJ), and transformed into TG1 or XL1-Blue competent *E. coli*. Individual clones capable of specific binding to the target antigen were isolated by conventional methods and then used to explore alternatives to centrifugation. Single colonies were picked into 0.1 ml cultures (2xYT supplemented with 2% glucose and 100 µg/ml Ampicillin) in a deep well 96-well plate and incubated at 37°C with shaking for 5-6 hours when cultures reached mid-log phase. Cultures received M13KO7 helper phage (~1 x 10<sup>9</sup> pfu in 5 µl) and were incubated for 1 hour at 37°C with shaking. A 50 µl aliquot was removed to a duplicate deep well plate and 1 ml of media (2xYT supplemented with 100 µg/ml Ampicillin, 50 µg/ml Kanamycin with or without 1 mM IPTG) was added to wells for overnight growth

at 30 °C. Polystyrene plates were coated with protein antigens (hIgG1 $\kappa$ , hIgG1 $\lambda$ , or rabbit IgG) (1-10  $\mu$ g/ml in 0.1 M sodium bicarbonate pH 9.6) overnight at 4 °C, blocked with 3% non-fat dry milk (NFM) in Tris buffered saline with 0.05% Tween-20 (pH 7.4, TBST), and washed 3x in TBST. Aliquots of bacterial culture were removed to a  
5 separate microtiter plate, or to wells in a 96-well filtration plate (MultiScreen plates from Millipore, Bedford MA), a vacuum was applied slowly, and filtrate collected in a microtiter plate. The remainder of the culture in the deep well plates was centrifuged at 1,725 x g (3,500 rpm in an Eppendorf 5804 equipped with an A2MTP rotor) for 30 minutes at room temperature (RT).

10 Aliquots (80  $\mu$ l), of clarified phage supernatant, filtrate, or uncleared bacterial culture were added to the ELISA plate and mixed with 20  $\mu$ l of 10% NFM/5x PBS directly in the wells. Plates were incubated without shaking for 1.5 hours at 37 °C, then washed 4x with TBST (using a Wellwash 4 MK2 platewasher, Labsystems). Horseradish peroxidase (HRP)-conjugated anti-M13 antibody(Amersham-Pharmacia  
15 Biotech, Piscataway, NJ) was diluted 1:5000 into 3% NFM/TBST and incubated in wells for 1 hour at 37 °C. Following 4 washes with TBST, 100  $\mu$ l of o-Phenylenediamine Dihydrochloride (OPD; Sigma, St. Louis MO) substrate was added to wells for approximately 5 minutes prior to stopping the reaction with 25  $\mu$ l 3N HCl. Plates were  
20 performed in duplicate and repeated 2 or 3 times with similar results.

The scFv display phage derived from HP6054 bind to the human lambda light chain antigen (in association with IgG1), but not to the kappa light chain (hIgG1 Kappa) or to Rabbit IgG (Figure 1A). Filtrate generated from the same culture yields an equivalent level of binding as observed for the supernatant. The uncleared bacterial  
25 culture demonstrated similar levels of binding to the immobilized antigen, indicating that removal of bacteria by time consuming centrifugation or costly filtration is not necessary. Furthermore, no increase in binding was observed to either of the two non-specific antigens tested, hIgG1 $\kappa$  and rabbit IgG.

Similar results were observed for phage displaying a Fab also derived from  
30 HP6054 (Figure 1B). Addition of IPTG adversely affects the binding of HP6054 scFv-phage (due to reduced bacterial growth and phage production) and increases the binding of HP6054 Fab-phage (due to increased Fab:gIII fusion production). Although the level of IPTG did affect the overall binding of the phage populations, there were no significant

differences in levels of binding observed when ELISA was performed directly on bacteria containing cultures, or cultures that were clarified by centrifugation or filtration.

5                   Example 2 - Sensitivity of phage ELISA is not impaired by the presence of bacteria.

                  In some applications, assay of a polyclonal population of phage producing bacteria would be necessary, e.g, library screening. For example, following several rounds of selection, one might wish to test the population by ELISA to verify that binding members have been selected and are present in the population. To demonstrate that  
10   bacteria remaining during the ELISA would not present a problem when low levels of binding were expected, we grew independent cultures prior to mixing them at various ratios and then performed the ELISA on the mixed cultures or centrifuge-clarified culture supernatants derived from the same mixtures. A mixture of cultures from two clones was  
15   used as a model system to simulate a polyclonal culture. Growth of a polyclonal culture involves competition between individuals, which affects the yield of specific phage. However, the bias that is introduced during polyclonal growth would exist regardless of the means of analysis of that culture.

*E.coli* carrying phagemid expressing scFv derived from HP6002 (recognizing hIgG2) or HP6025 (recognizing hIgG4) were grown overnight in the  
20   absence of IPTG. Cultures that attained different densities (OD<sub>600</sub> for HP6002 was 1.2 and 1.0, and HP6025 was 2.1 and 3.3 in two separate trials) were mixed on the basis of volume. Final volume ratios ranged from a 0.001 to 1000 of HP6025/HP6002. Aliquots of the mixed culture were compared to supernatants clarified by centrifugation in the phage ELISA. Figure 2 demonstrates that clarified phage supernatant and bacterial  
25   culture do not exhibit significant differences in binding at any of the ratios tested. Therefore, sensitivity of the ELISA does not appear to be compromised by the presence of bacteria during the binding of the phage an immobilized antigen.

                  Our results demonstrate the phage ELISAs can be performed directly on bacterial culture and that there is no need to clarify by centrifugation or filtration. We  
30   have successfully used culture from scFv (5 different antibodies) and Fab (2 different antibodies) display-phage in our ELISA.

                  Additionally, we have used this method for analysis of both peptides and proteins displayed on the major coat protein (gene VIII protein) of filamentous phage. To



date, no significant differences between culture and clarified supernatant have been observed for any display agents or antigen tested by this method.

Furthermore, this procedure has worked well with two common *E.coli* strains (TG1 cells and XL1-Blue) and overnight cultures of various densities (OD<sub>600</sub> from  
5 0.1 to 3.3).

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents,  
10 and patent applications cited herein are hereby incorporated by reference for all purposes.

**WHAT IS CLAIMED IS:**

- 1                   1.     A method for screening a population of replicable genetic packages  
2     to obtain replicable genetic packages that display on their surface a fusion protein that  
3     specifically binds to a target molecule, the method comprising:  
4                   contacting a target molecule with an uncleared cell culture, wherein said  
5     culture comprises:  
6                   (a) replicable genetic packages, each of which displays on its  
7     surface a fusion protein that comprises a surface-displayed replicable genetic package  
8     polypeptide and a potential binding polypeptide; and  
9                   (b) cells in which the replicable genetic packages were amplified;  
10                  wherein said replicable genetic packages that specifically bind to said  
11     target molecule form complexes that comprise the target molecule and the replicable  
12     genetic packages.
- 1                   2.     The method of claim 1, wherein said potential binding polypeptide  
2     is encoded by a member of a library of nucleic acid molecules.
- 1                   3.     The method of claim 2, wherein said nucleic acid molecules are  
2     cDNA molecules.
- 1                   4.     The method of claim 2, wherein said nucleic acid molecules are  
2     recombinant products.
- 1                   5.     The method of claim 1, wherein said method further comprises  
2     separating from said complexes cells and/or replicable genetic packages that do not  
3     specifically bind to said target molecule.
- 1                   6.     The method of claim 5, wherein at least 70% of the cells originally  
2     present in the culture are removed.
- 1                   7.     The method of claim 6, wherein at least 90% of the cells originally  
2     present in the culture are removed.
- 1                   8.     The method of claim 5, wherein said cells and unbound replicable  
2     genetic packages are separated from said complexes using aspiration.

- 1                   9.     The method of claim 5, wherein the method further comprises  
2     eluting said replicable genetic packages that specifically bind to said complexes.
- 1                   10.    The method of claim 1, wherein the presence of said complexes  
2     that comprise the target molecule and the replicable genetic packages is assessed by  
3     contacting the complexes with a detection reagent that binds to said replicable genetic  
4     packages.
- 1                   11.    The method of claim 10, wherein said detection reagent comprises  
2     an antibody.
- 1                   12.    The method of claim 10, wherein the complexes are contacted with  
2     the detection reagent in the presence of the cells.
- 1                   13.    The method of claim 1, wherein the replicable genetic packages are  
2     selected from the group consisting of bacteriophage and eukaryotic viruses.
- 1                   14.    The method of claim 1, wherein said target molecule is  
2     immobilized on a solid support.
- 1                   15.    The method of claim 14, wherein said solid support is selected  
2     from the group consisting of: a bead, a chip, a microtiter plate, a prokaryotic cell and a  
3     eukaryotic cell.
- 1                   16.    The method of claim 1, wherein said target molecule is selected  
2     from the group consisting of: a polypeptide, a nucleic acid, an RNA, a DNA, a small  
3     organic molecule, and a carbohydrate.
- 1                   17.    The method of claim 1, wherein said potential binding polypeptide  
2     is an antibody.
- 1                   18.    The method of claim 17, wherein said antibody is a scFv or a Fab.
- 1                   19.    The method of claim 1, wherein said method is performed on an  
2     automated laboratory workstation.
- 1                   20.    A composition comprising:

- 2 (a) population of replicable genetic packages, each of which  
3 displays on its surface a fusion protein that comprises a surface-displayed replicable  
4 genetic package polypeptide and a potential binding polypeptide; and  
5 (b) a complex that comprises a target molecule and one or more  
6 members of the population of replicable genetic packages that specifically bind to said  
7 target molecule; and  
8 (c) cells in which the replicable genetic packages were amplified.

1 21. The composition of claim 20, wherein said replicable genetic  
2 packages are selected from the group consisting of bacteriophage and eukaryotic viruses.

1 22. The composition of claim 20, wherein said target molecule is  
2 immobilized on a solid support.

1 23. The composition of claim 20, wherein said solid support is selected  
2 from the group consisting of: a bead, a chip, a microtiter plate, a prokaryotic cell and a  
3 eukaryotic cell.

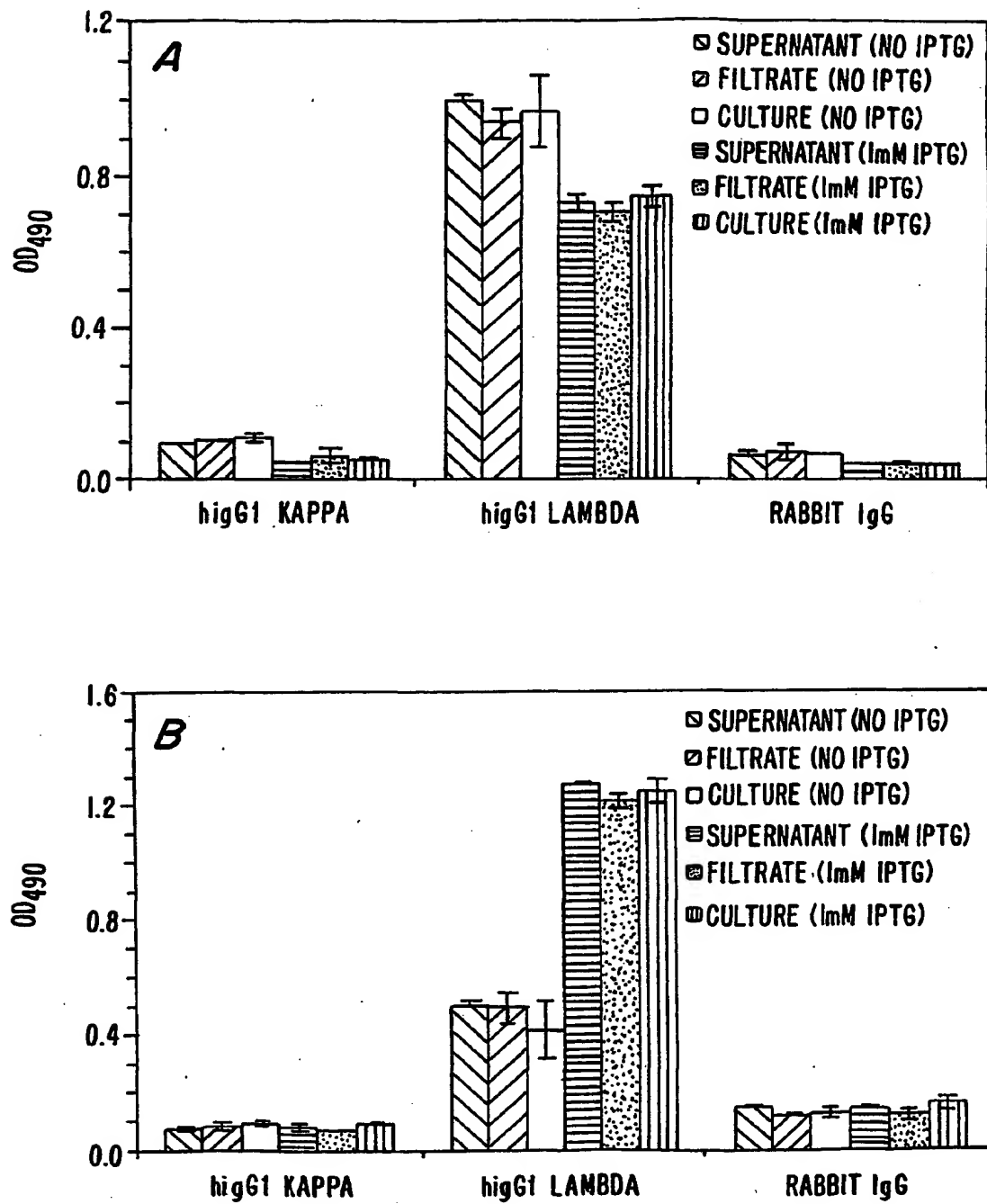
1 24. The composition of claim 20, wherein said target molecule is  
2 selected from the group consisting of: a polypeptide, a nucleic acid, an RNA, a DNA, a  
3 small organic molecule, and a carbohydrate.

1 25. The composition of claim 20, wherein said potential binding  
2 polypeptide is an antibody.

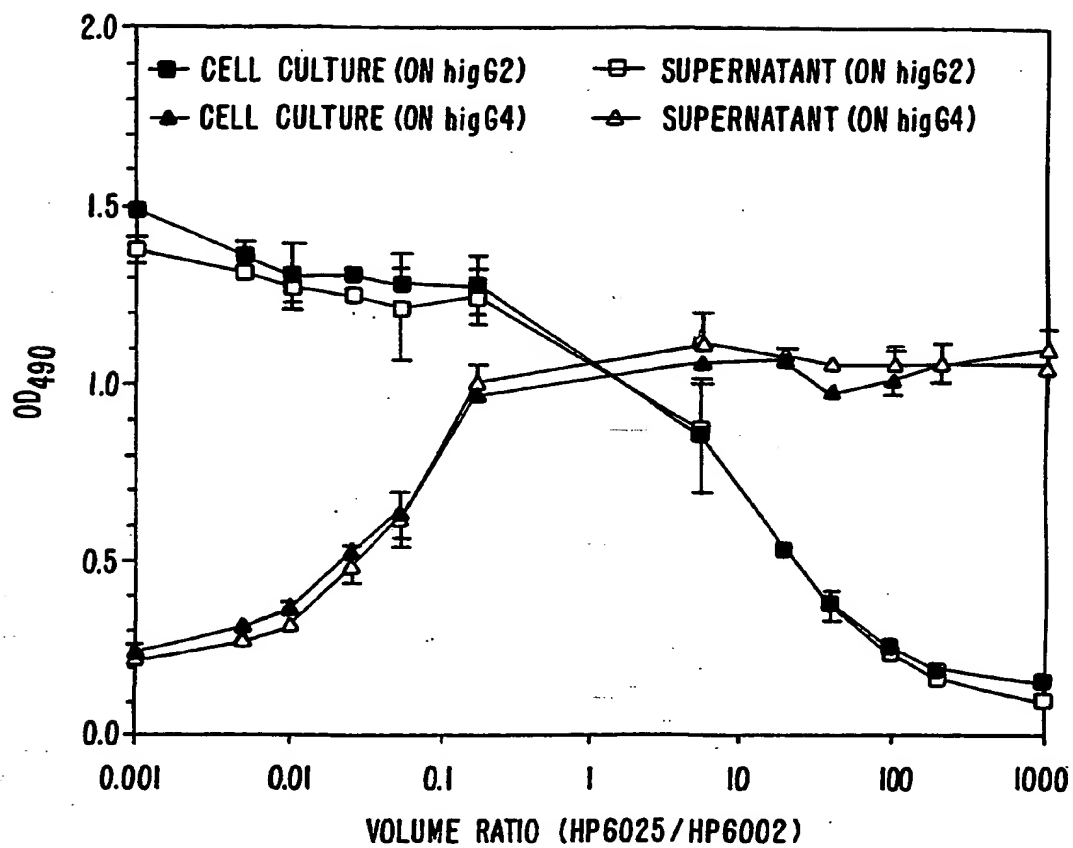
1 26. The composition of claim 25, wherein said antibody is a scFv or a  
2 Fab.

1 27. The composition of claim 20, wherein the composition further  
2 comprises a detection reagent that specifically binds to the replicable genetic packages.

1/2

**FIG. 1.**

2/2

**FIG. 2**

## SEQUENCE LISTING

SEQ ID NO:1: MCH1-G1R 48 mer

ATTGGCGCGCCTTATTAACAATCCCTGGGCACAATTTCTTGTCCACC

5 SEQ ID NO:2: MCH1-G2A 44 mer

ATTGGCGCGCCTTATTAACAGGGCTTGATTGTGGGCCCTCTGGG

SEQ ID NO:3: MCH1-G2B 45 mer

ATTGGCGCGCCTTATTAACAGGGGTTGATTGTTGAAATGGGCCCCG

10

SEQ ID NO:4: MHV-Back1 50 mer

TTATTACTCGCGGCCCCAGCCGGCCATGGCCGATGTGAAGCTTCAGGAGTC

SEQ ID NO:5: MHV-Back2 50 mer

15 TTATTACTCGCGGCCCCAGCCGGCCATGGCCCAGGTGCAGCTGAAGGAGTC

SEQ ID NO:6: MHV-Back3 50 mer

TTATTACTCGCGGCCCCAGCCGGCCATGGCCCAGGTGCAGCTGAAGCAGTC

20 SEQ ID NO:7: MHV-Back4 50 mer

TTATTACTCGCGGCCCCAGCCGGCCATGGCCCAGGTACTCTGAAAGAGTC

SEQ ID NO:8: MHV-Back5 51 mer

TTATTACTCGCGGCCCCAGCCGGCCATGGCCGAGGTCCAGCTGCAACAATC

25 T

SEQ ID NO:9: MHV-Back6 50 mer

TTATTACTCGCGGCCCCAGCCGGCCATGGCCGAGGTCCAGCTGCAGCAGTC

30 SEQ ID NO:10: MHV-Back7 51 mer

TTATTACTCGCGGCCCCAGCCGGCCATGGCCCAGGTCCAAGTGCAGCAGCC

T

SEQ ID NO:11: MHV-Back8 50 mer

TTATTACTCGCGGCCCAGCCGGCCATGGCCGAGGTGAAGCTGGTGGAGTC

SEQ ID NO:12: MHV-Back9 50 mer

5 TTATTACTCGCGGCCCAGCCGGCCATGGCCGAGGTGAAGCTGGTGGGAATC

SEQ ID NO:13: MHV-Back10 50 mer

TTATTACTCGCGGCCCAGCCGGCCATGGCCGATGTGAACTTGGAAAGTGTC

10 SEQ ID NO:14: MHV-For1 33 mer

ACCTGGCGCGCCTGCAGAGACAGTGACCAGAGT

SEQ ID NO:15: MHV-for1b 42 mer

15 ACCGCCTCCACCTGGCGCGCCTGCAGAGACAGTGACCAGAGT

SEQ ID NO:16: MHV-For2 33 mer

ACCTGGCGCGCCTGAGGAGACTGTGAGAGTGGT

SEQ ID NO:17: MHV-for2b 42 mer

20 ACCGCCTCCACCTGGCGCGCCTGAGGAGACTGTGAGAGTGGT

SEQ ID NO:18: MHV-For3 33 mer

ACCTGGCGCGCCTGAGGAGACGGTGACTGAGGT

25 SEQ ID NO:19: MHV-for3b 42 mer

ACCGCCTCCACCTGGCGCGCCTGAGGAGACGGTGACTGAGGT

SEQ ID NO:20: MHV-For4 33 mer

30 ACCTGGCGCGCCTGAGGAGACGGTGACCGTGGT

SEQ ID NO:21: MHV-for4b 42 mer

ACCGCCTCCACCTGGCGCGCCTGAGGAGACGGTGACCGTGGT



SEQ ID NO:22: MKV-back1 39 mer

TCTGGCGGTGGCGGATCGGATGTTTTGATGACCCAAACT

SEQ ID NO:23: MKV-Back2 39 mer

5 TCTGGCGGTGGCGGATCGGATATTGTGATGACGCAGGCT

SEQ ID NO:24: MKV-Back3 36 mer

TCTGGCGGTGGCGGATCGGATATTGTGATAACCCAG

10 SEQ ID NO:25: MKV-Back4 39 mer

TCTGGCGGTGGCGGATCGGACATTGTGCTGACCCAATCT

SEQ ID NO:26: MKV-Back5 39 mer

15 TCTGGCGGTGGCGGATCGGACATTGTGATGACCCAGTCT

SEQ ID NO:27: MKV-Back6 39 mer

TCTGGCGGTGGCGGATCGGATATTGTGCTAACTCAGTCT

SEQ ID NO:28: MKV-Back7 39 mer

20 TCTGGCGGTGGCGGATCGGATATCCAGATGACACAGACT

SEQ ID NO:29: MKV-Back8 39 mer

TCTGGCGGTGGCGGATCGGACATCCAGCTGACTCAGTCT

25 SEQ ID NO:30: MKV-Back9 39 mer

TCTGGCGGTGGCGGATCGCAAATTGTTCTCACCCAGTCT

SEQ ID NO:31: MKV-For1 38

30 GATGGTGATGTGCGGCCCGCCCGTTTCAGCTCCAGCTTG

SEQ ID NO:32: MKV-For2 40

GATGGTGATGTGCGGCCCGCCCGTTTATTTCCAGCTTGGT

SEQ ID NO:33: MKV-For3 39 mer

GATGGTGATGTGCGGCCGCGCGTTTATTTCCTCAACTTTG

SEQ ID NO:34: MKV-For4 40 mer

5 GATGGTGATGTGCGGCCGCGGATACAGTTGGTGCAGCATC

SEQ ID NO:35: MVH1 55 mer

CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTRMAGCTTCAGGAGTC  
AGGAC

10

SEQ ID NO:36: MVH2 55 mer

CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTSCAGCTKCAGCAGTC  
AGGAC

15 SEQ ID NO:37: MVH3 53 mer

CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGCAGCTGAAGSASTCA  
GG

SEQ ID NO:38: MVH4 55 mer

20 CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTGCAGCTTCAGGAGTCS  
GGAC

SEQ ID NO:39: MVH5 55 mer

25 CCTTTCTATGCGGCCAGCCGGCCATGGCCGARGTCCAGCTGCAACAGTC  
YGGAC

SEQ ID NO:40: MVH6 53 mer

30 CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGGTCCAGCTKCAGCAATCT  
GG

SEQ ID NO:41: MVH7 53 mer

CCTTTCTATGCGGCCAGCCGGCCATGGCCGAGSTBCAGCTGCAGCAGTCT  
GG

SEQ ID NO:42: MVH8 55 mer

CCTTTCTATGCGGCCCAGCCGGCCATGGCCCAGGTYCAGCTGCAGCAGTC  
TGGRC

5 SEQ ID NO:43: MVH9 53 mer

CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTYCAGCTYCAGCAGTC  
TGG

SEQ ID NO:44: MVH10 56 mer

10 CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTCCARCTGCAACAATCT  
GGACC

SEQ ID NO:45: MVH11 54 mer

15 CCTTTCTATGCGGCCCAGCCGGCCATGGCCCAGGTCCACGTGAAGCAGTC  
TGGG

SEQ ID NO:46: MVH12 52 mer

CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTGAASSTGGTGGAAATCT  
G

20 SEQ ID NO:47: MVH13 52 mer

CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAVGTGAAGYTGGTGGAGTC  
TG

SEQ ID NO:48: MVH14 55 mer

25 CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTGCAGSKGGTGGAGTC  
TGGGG

SEQ ID NO:49: MVH15 54 mer

CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAKGTGCAMCTGGTGCAGTC  
TGGG

30

SEQ ID NO:50: MVH16 53 mer

CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTGAAGCTGATGGARTC  
TGG

- SEQ ID NO:51: MVH17     55 mer  
CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTGCARCTTGTTGAGTCT  
GGTG
- 5    SEQ ID NO:52: MVH18     54 mer  
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GGA
- SEQ ID NO:53: MVH19     53 mer  
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GG
- SEQ ID NO:54: MVH20     54 mer  
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15   TGGG
- SEQ ID NO:55: MVH21     55 mer  
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GGCC  
20
- SEQ ID NO:56: MVH22     53 mer  
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GG
- 25   SEQ ID NO:57: MVH23     52 mer  
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- SEQ ID NO:58: MVH24     53 mer  
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SEQ ID NO:59: MVH25 53 mer

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5 SEQ ID NO:60: MVK1 38 mer

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TTACTCCGGTCCGCGGACATTGTGCTSACCCAGTCTCC

10

SEQ ID NO:62: MVK3 38 mer

TTACTCCGGTCCGCGGACATTGTGATGACTCAGTCTCC

SEQ ID NO:63: MVK4 38 mer

15 TTACTCCGGTCCGCGGACATTGTGCTMACTCAGTCTCC

SEQ ID NO:64: MVK5 38 mer

TTACTCCGGTCCGCGGACATTGTGYTRACACAGTCTCC

20 SEQ ID NO:65: MVK6 38 mer

TTACTCCGGTCCGCGGACATTGTRATGACACAGTCTCC

SEQ ID NO:66: MVK7 38 mer

TTACTCCGGTCCGCGGACATTMAGATRACCCAGTCTCC

25

SEQ ID NO:67: MVK8 38 mer

TTACTCCGGTCCGCGGACATTCAGATGAMCCAGTCTCC

SEQ ID NO:68: MVK9 38 mer

30 TTACTCCGGTCCGCGGACATTCAGATGACDCAGTCTCC

SEQ ID NO:69: MVK10 38 mer

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5     SEQ ID NO:71: MVK12     38 mer  
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10     SEQ ID NO:73: MVK14     38 mer  
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20     TTACTCCGGTCCGCGGACATTGTGATGACTCAGGCTAC

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25     SEQ ID NO:78: MVK19     37 mer  
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30     SEQ ID NO:80: MVK21     38 mer  
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SEQ ID NO:82: MVK23 38 mer

5 TTACTCCGGTCCGCGGACATTTTGCTGACTCAGTCTCC

SEQ ID NO:83: MVK24 38 mer

TTACTCCGGTCCGCGGACATTGTAATGACCCAATCTCC

10 SEQ ID NO:84: MVK25 38 mer

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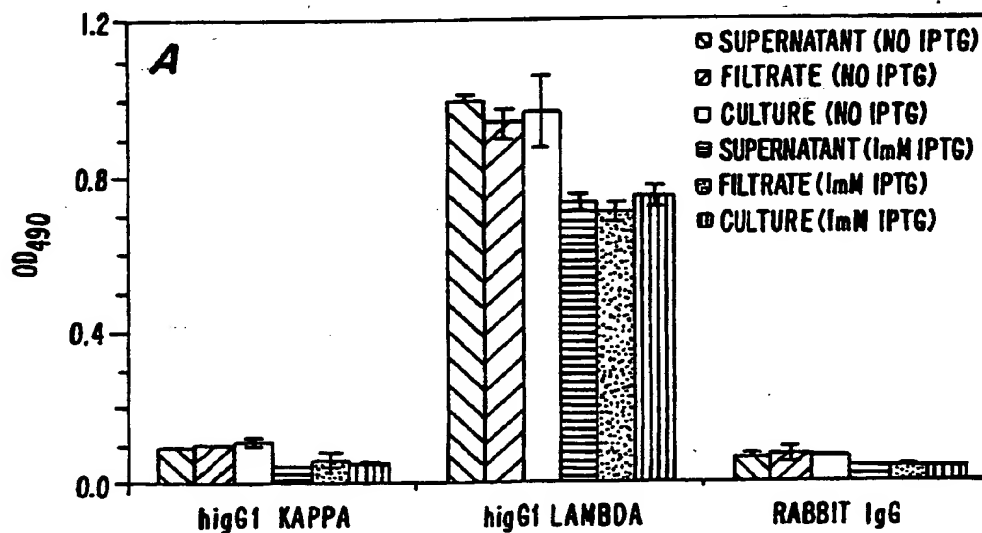
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- (71) Applicant (for all designated States except US): ZY-OMYX, INC. [US/US]; 26101 Research Road, Hayward, CA 94545 (US).
- (72) Inventors; and
- (73) Inventors/Applicants (for US only): NOCK, Steffen [DE/US]; 3269 Glenwood Avenue, Redwood City, CA 94062 (US). KASSNER, Paul, D. [US/US]; 247 W. 40th Avenue, San Mateo, CA 94403 (US).
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— with international search report

[Continued on next page]

(54) Title: SCREENING OF PHAGE DISPLAYED PEPTIDES WITHOUT CLEARING OF THE CELL CULTURE



(57) Abstract: This invention provides methods for screening populations of phage-displayed polypeptides that are particularly well-suited for high-throughput screening. The methods do not require the clearing of cells from a culture used to obtain the population of phage or other replicable genetic packages. Accordingly, the invention provides methods for forming complexes between a replicable genetic package displaying a polypeptide fusion and a target molecule in an uncleared cell culture containing replicable genetic package. Compositions made up of an uncleared cell culture containing replicable genetic packages displaying a polypeptide fusion and a target molecule are provided in the invention as well.

WO 01/94950 A3





— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**(88) Date of publication of the international search report:**  
10 May 2002

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International Application No  
PCT/US 01/18421

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, SEQUENCE SEARCH, BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	PHIPPS M LISA ET AL: "Detection of antibody display phage without clearing of bacterial culture." BIOTECHNIQUES, vol. 29, no. 4, 2000, pages 737-740, XP002191575 ISSN: 0736-6205 the whole document --- -/--	1-27

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl  
Fax: (+31-70) 340-3016

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Hornig, H

## INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HAARD DE H J ET AL: "A LARGE NON-IMMUNIZED HUMAN FAB FRAGMENT PHAGE LIBRARY THAT PERMITS RAPID ISOLATION AND KINETIC ANALYSIS OF HIGH AFFINITY ANTIBODIES"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US;</p> <p>vol. 274, no. 26,</p> <p>25 June 1999 (1999-06-25), pages 18218-18230, XP002931203</p> <p>ISSN: 0021-9258</p> <p>cited in the application</p> <p>the whole document</p> <p>---</p>	
A	<p>SHEETS MICHAEL D ET AL: "Efficient construction of a large nonimmune phage antibody library: The production of high-affinity human single-chain antibodies to protein antigens."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES,</p> <p>vol. 95, no. 11, 26 May 1998 (1998-05-26), pages 6157-6162, XP002191576</p> <p>May 26, 1998</p> <p>ISSN: 0027-8424</p> <p>cited in the application</p> <p>the whole document</p> <p>---</p>	
A	<p>WO 00 31246 A (SBLATTERO DANIELE ; S I S S A SCUOLA INTERNAZIONALE (IT); BRADBURY A)</p> <p>2 June 2000 (2000-06-02)</p> <p>the whole document</p> <p>---</p>	
A	<p>GRIFFITHS A D ET AL: "ISOLATION OF HIGH AFFINITY HUMAN ANTIBODIES DIRECTLY FROM LARGE SYNTHETIC REPERTOIRES"</p> <p>EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB,</p> <p>vol. 13, no. 14,</p> <p>15 July 1994 (1994-07-15), pages 3245-3260, XP000455240</p> <p>ISSN: 0261-4189</p> <p>the whole document</p> <p>---</p>	
A	<p>WO 95 34648 A (DADE INT INC)</p> <p>21 December 1995 (1995-12-21)</p> <p>the whole document</p> <p>---</p>	
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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 01/18421

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KREBBER CLAUS ET AL:            "Selectively-infective phage (SIP): A            mechanistic dissection of a novel in vivo            selection for protein-ligand interactions"            JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB,            vol. 268, no. 3, 1997, pages 607-618,            XP002151869            ISSN: 0022-2836            the whole document -----</p>	

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Information on patent family members

International Application No

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